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Combination of ATP-bioluminescence and PNA probes allows rapid total counts and identification of specific microorganisms in mixed populations

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Abstract

We have combined ATP-dependent bioluminescence with a novel chemiluminescent in situ hybridization (CISH) method using peroxidase-labeled peptide nucleic acid (PNA) probes targeting species-specific rRNA sequences to provide total counts and subsequent identification of specific microorganisms. Both methods are applied to the same membrane filter following a short incubation time and both methods provide results in the form of spots of light that are captured by the MicroStar™ detection system. Each spot of light represents individual micro-colonies detected by either ATP bioluminescence or PNA CISH. This new concept is particularly intended for in process and quality control of non-sterile products to rapidly provide total counts as well as presence/absence of specific indicators and/or pathogens in non-sterile, filterable samples.

Keywords: ATP; Bioluminescence; Incubation

1. Introduction

Non-sterile products manufactured for human consumption and health care are often allowed to contain a specified maximum microbial content, but must be free of certain pathogens and/or indicator microorganisms. Quality control of in-process material and final products typically involves bioburden analysis yielding the total number of colony-forming units (total counts) followed by specific presence/absence tests for specific pathogenic or indicator microorganisms of interest. *Escherichia coli* and *Pseudomonas aeruginosa* are examples of important indicators that are monitored in a variety of products, including non-sterile pharmaceuticals, beverages, and water (De La Rosa et al., 1995; Clesceri et al., 1998; Jimenez et al., 2000). Both national and international recommendations and regulations have been issued (Warburton, 1992; European Community, 1980; United States Pharmacopeia, 2000).

Current standard methods are based on detection, enumeration, and presumptive identification from visible growth on various media followed by final identification by biochemical analysis (Clesceri et

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al., 1998). Procedures typically take a minimum of 2–3 days depending on the microorganisms being monitored resulting in delays of product release that have significant impact on the manufacturing process and inventory management. The possible economic benefits of using rapid methods are obvious and various rapid microbiology methods have been developed. However, none of these rapid methods provide both total counts as well as specific identification of certain indicator organisms.

Bioburden analysis of filterable samples based on ATP-bioluminenscence in the form of the MicroStar™ Rapid Microbiology Detection Reagents (Millipore, Bedford, MA) provides rapid detection and enumeration of microorganisms in filterable samples following a short growth step (Upperman et al., 1999; Takahashi et al., 2000). Each colony-forming unit is determined by a spot of bioluminescence captured by the MicroStar Detection System, a highly sensitive camera system (Yasui and Yoda, 1997). However, the method does not provide the identity of the detected microorganisms. The use of the MicroStar Reagents and System for non-sterile samples is, therefore, limited, as it must be accompanied by slower, traditional growth-based methods for detection and identification of the specific indicators and/or pathogens.

Chemiluminescent in situ hybridization (CISH) uses peroxidase-labeled peptide nucleic acid (PNA) probes targeting species-specific rRNA sequences for rapid detection, identification and enumeration following membrane filtration and has recently been described for a variety of bacterial species, including *P. aeruginosa* and *E. coli* (Stender et al., 2000; Perry-O'Keefe et al., 2001). The PNA CISH assay format provides results in the form of spots of light representing individual cfu that can be captured by the MicroStar Detection System (Millipore) (Stender et al., 2000). However, the PNA CISH method by itself only detects specific microorganisms hybridizing with the PNA probe and does not provide total counts.

In this study, we combined these two methods, first using ATP-bioluminescence for rapid total count determination and subsequently performing specific identification of an indicator microorganism with the PNA CISH method on the same membrane filter. Comparing images of the spots of light obtained by each of the two methods enabled rapid identification and enumeration of specific indicator organisms in mixed populations in conjunction with total counts.

2. Materials and methods

2.1. Sample preparation

E. coli (8739), *Salmonella choleraesuis* (29946) and *P. aeruginosa* (9027) were obtained from the American Type Culture Collection, Manassas, VA. The strains were propagated in either LB broth (Sigma, St. Louis, MO) or tryptic soy broth (Becton Dickinson, Sparks, MD) at 30–35°C. Prior to membrane filtration, each strain was diluted to approximately 50 cfu/sample volume in either Fluid A (Millipore) or phosphate-buffered saline (PBS). Each sample was filtered through a 0.45 μm RMHV membrane filter (Millipore). The membrane filter was aseptically transferred using forceps to a petridish containing tryptic soy agar (Becton Dickinson) and incubated at 35°C prior to analysis. Incubation times varied as described below.

2.2. ATP bioluminescence

Detection of micro-colonies by ATP-dependent bioluminescence was performed using the MicroStar Rapid Microbiology Detection Reagents (Millipore) according to the manufacturer's instructions. Briefly, the membrane filter was removed from the agar plate and allowed to dry. Initially, the membrane filter was sprayed with the ATP-releasing reagent. Once dry, the membrane filter was sprayed with ATP-bioluminescence reagent and immediately imaged for 2 min by the MicroStar™ Detection System (Millipore).

2.3. Chemiluminescent in situ hybridization using PNA probes (PNA CISH)

PNA CISH was performed as previously described with minor modifications (Stender et al., 2000). Briefly, micro-colonies were fixed to the membrane filter by placing it on a cellulose pad soaked with 1.5 ml of fixation solution (0.35% (v/v) glutaraldehyde (Sigma), 0.01% (w/v) urea–H_2O_2 (Sigma), 5 mM NaN_3 in 90% (v/v) denatured

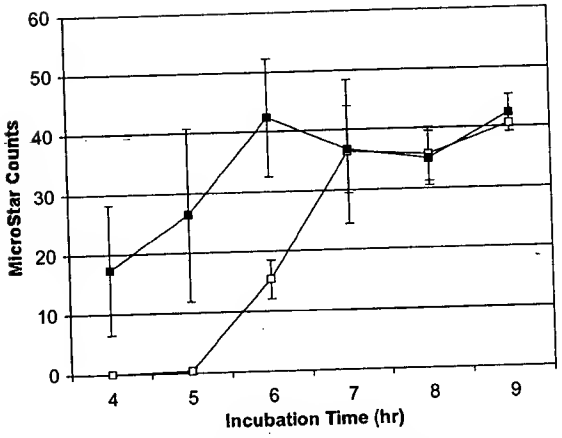

Fig. 1. Enumeration of *P. aeruginosa* micro-colonies by ATP-bioluminescence (□) and *P. aeruginosa* PNA CISH (■) as a function of incubation time. Each data point is an average of three determinations and the error bars represents the standard deviation.

Table 1
Comparison of number of light spots obtained by ATP-bioluminescence and PNA CISH for *P. aeruginosa*
The numbers are means of triplicate determinations.

	PNA CISH positive	PNA CISH negative	Σ
ATP positive	32.3	3.33	35.6
ATP negative	2.67	–	2.67
Σ	35.0	3.33	38.3

ethanol) for 5 min. Hybridization was performed for 30 min at 50°C in a covered Petrislide (Millipore) using 1.5 ml of 1–3 nM soybean peroxidase (SBP)-labeled PNA probes in hybridization solution (25 mM Tris (pH 9.5), 50% (v/v) formamide, 2% (w/v) polyvinolpyrrolidone, 0.7% (v/v) Tween 20, 1%(w/v) casein (Sigma), 0.1 M NaCl, and 5 mM EDTA). SBP-labeled PNA probes targeting 16S rRNA of *P. aeruginosa* (Pse16S32/SBP), *E. coli* (Eco16S06/SBP) and all eubacteria (BacUni1/SBP) were obtained from Boston Probes, Bedford, MA. Excess probe was removed by washing the membrane filter four times at 7-min intervals at 50°C in Wash Solution (10 mM CAPSO (Sigma) (pH 10.0), 0.2% (v/v) Tween 20). Binding of probe was visualized by placing the filter for 2 min in a mixture of 150 μl Luminonol/Enhancer and 150 μl Stable Peroxide, both part of the SuperSignal Chemiluminescent Substrate (Pierce Chemical, Rockford, IL) supplemented with 10 mM H_2O_2. Excess substrate was removed and the membrane filter was imaged

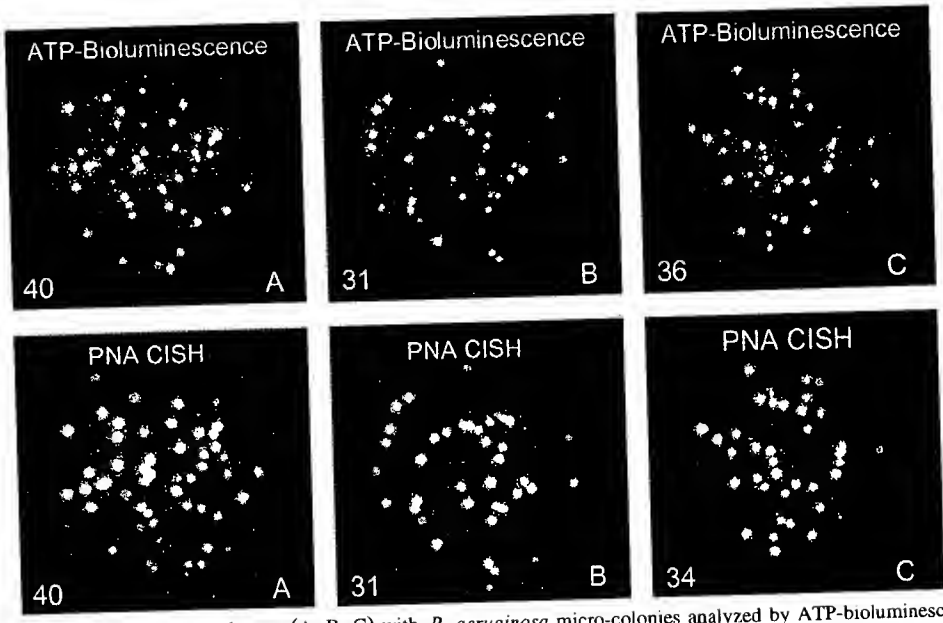

Fig. 2. MicroStar images of three membranes (A, B, C) with *P. aeruginosa* micro-colonies analyzed by ATP-bioluminescence and by *P. aeruginosa* PNA CISH after 8 h of incubation. Counts are listed in the lower left corner of each image.

for 5–120 s using the MicroStar Detection System (Millipore).

2.4. Interpretation of results

Spots of light on each image were enumerated using the RMDS Control Program, v. 1.17 (Hamamatsu Photonics K.K., Japan) included in the MicroStar Detection System (Millipore).

3. Results and discussion

3.1. Determination of incubation time

It is crucial that the assay conditions are optimized to yield correlation between the two technologies such that the presence of the specific organism is detected by both technologies. The most important parameter in this respect is the growth time prior to analysis. Fig. 1 shows the results obtained by ATP-bioluminescence and by subsequent PNA CISH analysis as a function of the growth time. Correlation between the two technologies occurred after minimum 7 h of growth. Although the PNA CISH method reached a plateau after 6 h of growth, ATP-bioluminescence required additional 1 h of incubation after which the counts correlated between the two assays. It was decided to use a growth time of 8 h for the following experiments with *P. aeruginosa*.

For the three membranes shown in Fig. 2, individual spots detected by ATP-bioluminescence and PNA CISH were compared (Table 1). This comparison revealed that 91% of the spots detected using ATP were also detected by PNA CISH. Similarly, 92% of the spots identified by PNA CISH were also identified using ATP-bioluminescence. The remaining spots (9% ATP-positive, PNA CISH-negative and 8% ATP-negative, PNA CISH-positive) were partly explained by inconsistent counts due to spots being close to the membrane perimeter or spots being close to each other. Of the very few remaining discrepant spots, ATP-positive, CISH-negative spots may be explained by contamination. ATP-negative, CISH-positive spots may be due the higher sensitivity of PNA CISH as data in Fig. 1 showed that micro-colonies are detected earlier with PNA CISH than with ATP-bioluminescence. Alternatively, they may sim-

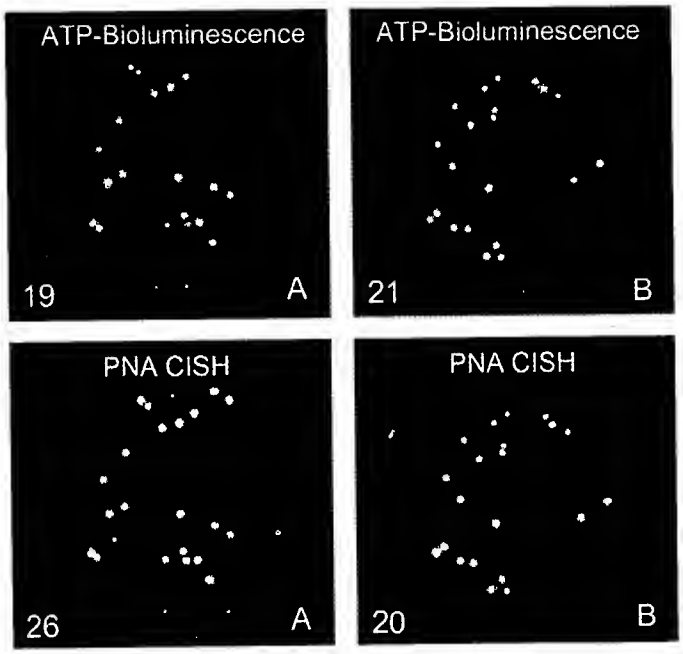

Fig. 3. MicroStar images of *E. coli* (A) and *S. choleraesuis* (B) analyzed by ATP-bioluminescence and Eubacterium PNA CISH after 5 h of incubation. Counts are listed in the lower left corner of each image.

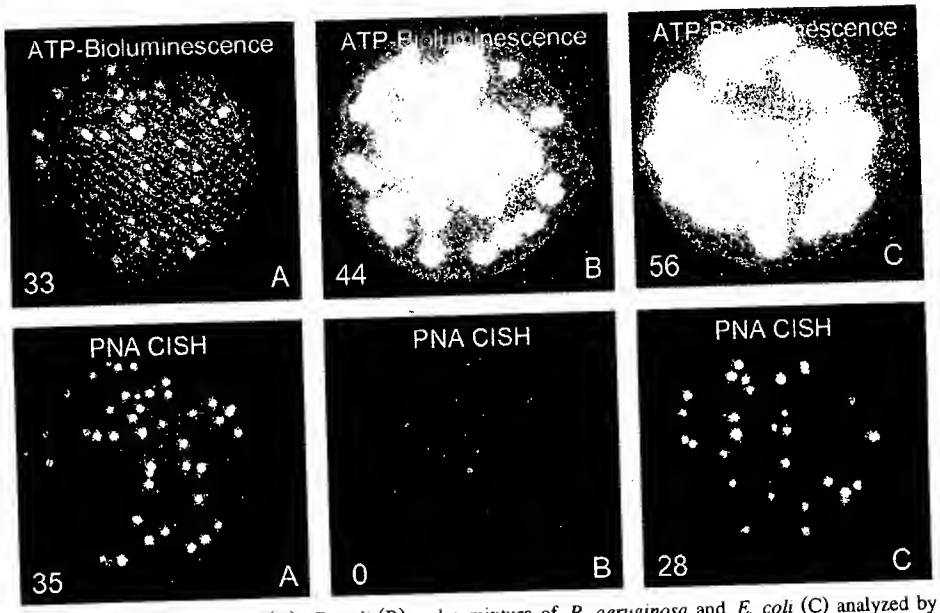

Fig. 4. MicroStar images of *P. aeruginosa* (A), *E. coli* (B) and a mixture of *P. aeruginosa* and *E. coli* (C) analyzed by ATP-bioluminescence and *P. aeruginosa* PNA CISH after 8 h of incubation. Counts are listed in the lower left corner of each image.

ply be unexplained, false positive spots (background). The level of unexplained spots was determined by the analysis of negative samples (100 ml Fluid A). An average of approximately 0 spots by ATP-bioluminescence and 1 spot by *P. aeruginosa* PNA CISH was found.

For *E. coli* and *S. choleraesuis* it was found that 5 h of incubation was sufficient to obtain equivalent

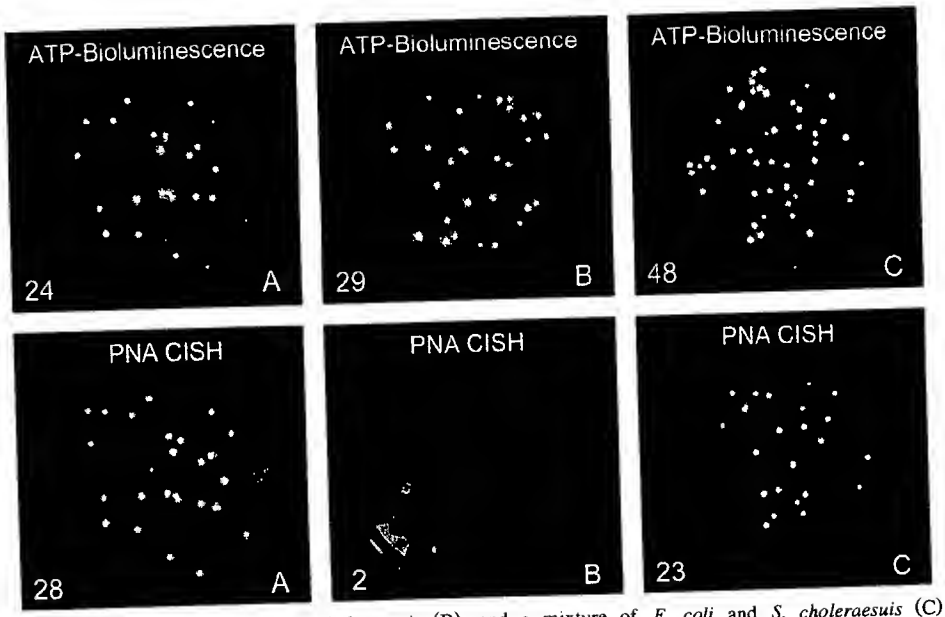

Fig. 5. MicroStar images of *E. coli* (A), *S. choleraesuis* (B), and a mixture of *E. coli* and *S. choleraesuis* (C) analyzed by ATP-bioluminescence and *E. coli* PNA CISH after 5 h of incubation. Counts are listed in the lower left corner of each image.

counts with both methods. This was based on analysis of *S. choleraesuis* and *E. coli* by ATP-bioluminescence followed by PNA CISH using BacUni1/SBP, a PNA probe targets all eubacterium species (Fig. 3). In this case, ATP-bioluminescence and PNA CISH generated almost identical images.

3.2. Identification of P. aeruginosa in mixed populations

Fluid A samples (100 ml) spiked with either *P. aeruginosa*, *E. coli*, or a mixture of both were filtered and incubated for 8 h on TSA at 35°C. The membranes were then analyzed by ATP-bioluminescence and subsequently by PNA CISH using a *P. aeruginosa*-specific PNA probe (Fig. 4). Again, almost identical images were obtained for *P. aeruginosa*, whereas *E. coli* was detected by ATP-bioluminescence only. As expected for the mixture of *P. aeruginosa* and *E. coli*, ATP-bioluminescence generated counts that were approximately two times higher than counts obtained with *P. aeruginosa* PNA CISH. The combination of the two technologies, thus, provided total counts of *P. aeruginosa* and *E. coli* and subsequent identification and enumeration of *P. aeruginosa* independently of the presence of *E. coli*.

3.3. Identification of E. coli in mixed populations

S. choleraesuis, *E. coli*, and a mixture of both were prepared as described above and analyzed by ATP-bioluminescence and *E. coli* PNA CISH. Images in Fig. 5 illustrate that *E. coli* was specifically identified and enumerated in the presence of *S. choleraesuis* by *E. coli* PNA CISH along with total counts based on ATP-bioluminescence. The count of two spots by *E. coli* PNA CISH with *S. choleraesuis* are examples of unexplained spots.

4. Conclusion

The lack of identification capabilities of rapid membrane filtration methods has so far limited their utility in bioburden testing. This study demonstrates that a membrane filtration based, ATP-bioluminescence method for rapid detection and enumeration of microorganisms can be combined with subsequent PNA CISH to identify and enumerate specific microorganisms on the exact same membrane filter. Thus, employing molecular technologies to provide identification dramatically expands the utility of the current platform developed for rapid total counts. The combination of assays can be used to rapidly determine total counts and absence of specific microorganisms in non-sterile samples prior to release resulting in faster production cycles and lower inventories.

The PNA CISH technology utilizes PNA probes targeting species-specific rRNA sequences allowing microorganisms to be identified in a single assay (Delong et al., 1989; Amann et al., 1995, Stender et al., 1999). Although, the specificity of the PNA probes was not addressed in this study, they have been extensively evaluated in separate publications (Stender et al., 2000, 2001; Perry-O'Keefe et al., 2001). Likewise, the correlation of the number of light spots to standard colony counts following sustained growth has been shown to be 90–100% (Stender et al., 2000, 2001; Perry-O'Keefe et al., 2001; Millipore, unpublished data).

Sequential use of ATP-bioluminescence and PNA CISH on the same membrane increases time to results by approximately 90 min relative to performing the two assays on duplicate membranes in parallel, however, significant advantages are gained. Firstly, only one membrane per sample must to be processed. Secondly, membranes having 0 cfu by ATP bioluminescence do not need further analysis by PNA CISH. Moreover, the use of two different detection technologies on the same membrane reduces the risk of false positive spots of light, as each micro-colony of the particular microorganism has to be detected by both ATP-bioluminescence and PNA CISH (i.e. the spots must align in the two methods). This is particularly important in many quality control applications where the prevalence of the indicator organism is very low.

Although, this study used *P. aeruginosa* and *E. coli* as target organisms the concept presumably can be applied to other microorganisms. Particularly, since both the ATP-bioluminescence and PNA CISH technologies have been evaluated on a range of different types of bacteria and yeast, including both

gram-positive and gram-negative bacteria (Perry-O'Keefe et al., 2001; Millipore, unpublished data).

In summary, the combination of ATP-bioluminescence and PNA CISH as presented in this study enables the use of rapid microbiological methods for various quality control procedures for non-sterile, filterable samples by providing both total counts as well as presence/absence of a particular indicator microorganism.

References

Amann, R.I., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and in situ detection of individual cells without cultivation. Microbiol. Rev. 59, 143–169.

Clesceri, L.S., Greenberg, A.E., Eaton, A.D., 1998. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington, DC.

De La Rosa, M.C., Medina, M.R., Vivar, C., 1995. Microbiological quality of pharmaceutical raw materials. Pharm. Acta Helv. 70, 227–232.

Delong, E.F., Wickham, G.S., Pace, N.R., 1989. Phylogenic stains: ribosomal RNA-based probes for the identification of single cells. Science 243, 1360–1363.

European Community, 1980. Council Directive no. 80/777/EEC of 15 July 1980 on the approximation of the laws of the member states relating to the exploitation and marketing of natural mineral water. Off. J. Eur. Communities L229, 1–10.

Jimenez, L., Smalls, S., Ignar, R., 2000. Use of PCR analysis for detecting low levels of bacteria and mold contamination in pharmaceutical samples. J. Microbiol. Methods 41, 259–265.

Perry-O'Keefe, H., Stender, H., Broomer, A., Oliveira, K., Coull, J., Hyldig-Nielsen, J.J., 2001. Filter-based PNA in situ hybridization for rapid detection, identification and enumeration of specific microorganisms. J. Appl. Microbiol. 90, 180–189.

Stender, H., Lund, K., Petersen, K.H., Rasmussen, O.F., Hongmanee, P., Miorner, H., Godtfredsen, S.E., 1999. Fluorescence in situ hybridization assay using peptide nucleic acid probes for differentiation between tuberculous and nontuberculous mycobacterium species in smears of mycobacterium cultures. J. Clin. Microbiol. 37, 2760–2765.

Stender, H., Broomer, A., Oliveira, K., Perry-O'Keefe, H., Hyldig-Nielsen, J.J., Sage, A., Young, B., Coull, J., 2000. Rapid detection, identification, and enumeration of *Pseudomonas aeruginosa* in bottled water using peptide nucleic acid probes. J. Microbiol. Methods 42, 245–253.

Stender, H., Broomer, A.J., Oliveira, K., Perry-O'Keefe, H., Hyldig-Nielsen, J.J., Sage, A., Coull, J., 2001. Rapid detection, identification, and enumeration of *Escherichia coli* in municipal water by chemiluminescent in situ hybridization. Appl. Environ. Microbiol. 67, 142–147.

Takahashi, T., Nakakita, Y., Watari, J., Shinotsuka, K., 2000. Application of a bioluminescence method for the beer industry: sensitivity of MicroStar™-RMDS for detecting beer-spoilage bacteria. Biosci., Biotechnol., Biochem. 64, 1032–1037.

United States Pharmacopeia, 2000. USP 2r/NF 1.

Upperman, S.D., Young, B.E.S., Presente, E.M., 1999. Rapid enumeration of microorganisms in purified water. O-14. Abstract. Annual Meeting of the American Society of Microbiology, Washington, DC.

Warburton, D.W., 1992. A review of the microbiological quality of bottled water sold in Canada: Part 2. The need for more stringent standards and regulations. Can. J. Microbiol. 39, 158–168.

Yasui, T., Yoda, K., 1997. Imaging of Lactobacillus bevis single cells and microcolonies without a microscope by ultrasensitive chemiluminescent enzyme immunoassay with a photon-counting television camera. Appl. Environ. Microbiol. 63, 4528–4533.